VIROLOGY 249, 231–237 (1998)
ARTICLE NO. VY989345

Mushroom Bacilliform Virus RNA: The Initiation of Translation at the 5′ End of the Genome and Identification of the VPg

Peter A. Revill,[1] Andrew D. Davidson, and Peter J. Wright

Department of Microbiology, Monash University, Wellington Rd., Clayton, Victoria 3168, Australia

Received May 18, 1998; returned to author for revision June 25, 1998; accepted July 22, 1998

Mushroom bacilliform virus (MBV) is often found in cultivated mushrooms (*Agaricus bisporus*) with La France disease. MBV has a 4-kb ssRNA genome of positive-sense encoding four major open reading frames (ORFs). The arrangement of ORFs at the 5′ end of the genome and the deduced amino-acid sequences of two of the putative gene products (protease and RNA-dependent RNA polymerase) show remarkable similarity to some plant viruses, particularly subgroup II luteoviruses. We show that this similarity extends to the translation strategy at the 5′ end of the genome, the presence of a genome-linked protein (VPg), and the location of the VPg downstream of the protease motifs in the polypeptide encoded by ORF2.

INTRODUCTION

Mushroom bacilliform virus (MBV) is the sole member of the family Barnaviridae (Romaine, 1995). The virion is a 19 × 50-nm bacilliform particle encapsidating a ssRNA positive-sense monopartite genome of 4 kb (Tavantzis *et al.,* 1980, 1983). MBV is associated with La France disease (LFd) in the cultivated mushroom, *Agaricus bisporus,* although the role of the virus in causing the disease is unclear (Hollings, 1962; Hollings *et al.,* 1968; van Zaayen, 1979). A second virus with an icosahedral shape and a genome of dsRNA, La France infectious virus (LIV), is strongly implicated as the primary cause of the disease (Goodin *et al.,* 1992; van der Lende *et al.,* 1994). MBV is not detected in healthy mushrooms and yet is not detected in all cases of LFd (van Zaayen, 1979; Romaine and Schlagnhaufer, 1995).

The MBV genome has been sequenced and contains four major ORFs (1–4), encoding polypeptides of M_r 20,000, 73,000, 47,000, and 22,000, respectively (Fig. 1) (Revill *et al.,* 1994). The organisation of the MBV ORFs and their deduced amino-acid sequences shows some similarity to plant viruses, particularly subgroup II luteoviruses and sobemoviruses (Revill *et al.,* 1994; Revill, 1995; Mäkinen *et al.,* 1995).

ORF1 of MBV begins with AUG at nt 61 and overlaps ORF2, which commences with AUG at nt 68. ORF1 also contains a second in-frame AUG codon at nt 100. It has not been determined which of these codons is used to initiate translation. This is of some interest, as the preferred consensus sequence (xA/GxxAUGG) for initiation of translation (Lütcke *et al*, 1987; Kozak, 1989; Matthews, 1991) is not present for the first AUG in ORF1 and is only partially satisfied for the first ORF2 AUG and the second AUG in ORF1 To elucidate the translation strategy at the 5′ end of the genome, genomic-length cDNA of MBV was assembled, then transcribed and translated *in vitro*.

We also investigated whether MBV RNA has a genome-linked protein (VPg) at its 5′ terminus. Recently, van der Wilk *et al.* (1997) identified a VPg for potato leafroll virus (PLRV), a subgroup II luteovirus. The VPg was located in the ORF1 polyprotein (analogous to MBV ORF2), downstream of the serine protease motifs. This genome arrangement (protease-VPg-polymerase) is unusual, as all single-stranded RNA viruses for which a VPg has been identified have the arrangement VPg-protease-polymerase (Koonin and Dolja, 1993; van der Wilk *et al.,* 1997). Using radiolabelling and Edman degradation, we have identified the VPg for MBV and shown that it is located in a similar position on the genome to PLRV, downstream of the protease motifs in the ORF2 polyprotein.

RESULTS

VPg

The iodination with [^{125}I] of a sample containing purified viral RNA and associated protein, followed by RNase treatment, electrophoresis, and autoradiography, revealed two products of M_r 16,000 (a) and M_r 15,000 (b) (Fig. 2, lane 1). Proteinase K treatment removed both products (Fig. 2, lane 2), indicative of the presence of polypeptide. Edman degradation of unlabeled protein associated with purified RNA yielded an unambiguous single sequence of SEETDRYART, suggesting that the polypeptide(s) resolved as the two bands in Fig. 2 had a common N terminus and represented forms of VPg. The

[1] To whom reprint requests should be addressed. Fax: 61399054811, E-mail: peter.revill@med.monash.edu.au.

0042-6822/98 $25.00

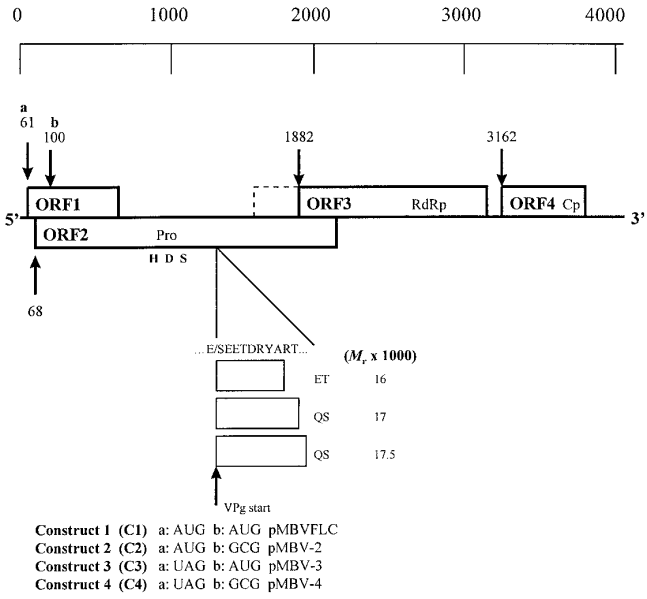

FIG. 1. The MBV genome arrangement showing the predicted coding regions for the N-terminal VPg sequence, coat protein (Cp), and putative serine protease (Pro) and RNA-dependent RNA polymerase (RdRp) domains. Boxes with solid lines indicate the ORFs, each commencing with the first AUG. The positions of the first and second AUGs in ORF1 are designated a and b, respectively. ORF3 upstream of the first ORF3 AUG is indicated by dashed lines. The constructs (C1–4) containing changes to the first two AUGs in ORF1 are listed at the bottom of the figure. Putative E/T and Q/S serine protease cleavage sites located downstream of the VPg N terminus, and the approximate M_r of products derived from cleavage at these sites, are shown. The approximate location of the putative serine protease catalytic triad upstream of the VPg sequence is also marked (H D S).

multiple forms of VPg may have resulted from heterogeneity in the length of the VPg itself or the attachment of residual ribonucleotides.

The N-terminal sequence SEETDRYART is identical to the deduced amino-acid sequence encoded by nucleotides 1334–1363 in MBV ORF2 (residues 464–473), demonstrating that the VPg is encoded downstream of the putative serine protease motifs in ORF2 (Fig. 1). The N-terminal portion of the MBV VPg sequence showed no similarity to the PLRV VPg sequence (van der Wilk *et al.,* 1997).

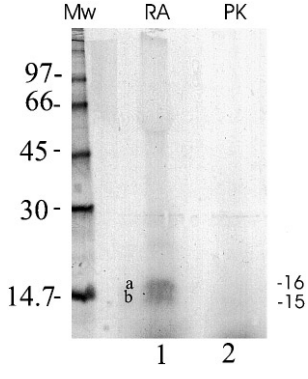

FIG. 2. Analysis by gel electrophoresis of the iodinated MBV VPg. Samples were either treated with RNase A (RA, lane 1), or proteinase K (PK, lane 2). The sizes of ^{14}C-labeled molecular weight markers are indicated (Mw).

In vitro translation

Translation of the full-length MBV RNA produced two major polypeptides of approximate M_r 21,000 and 72,000, and numerous minor polypeptides ranging in size from M_r 18,000 to 60,000 (estimated on gel mobility; Fig. 3A, lane 1). Similar results were obtained using either rabbit reticulocyte lysates or wheat germ extracts for the translation experiments. Removal of the first AUG only in

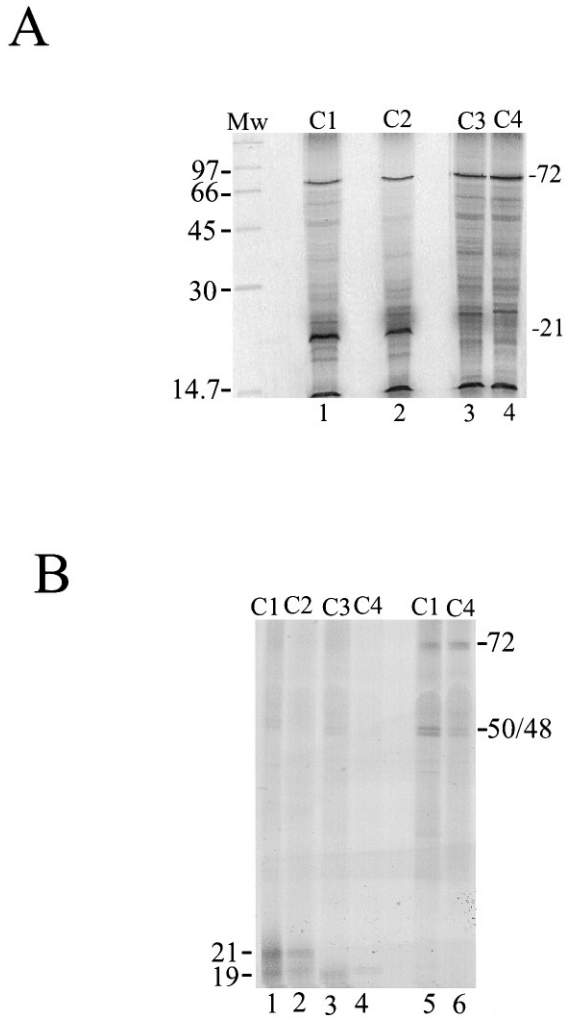

FIG. 3. (A) Analysis by gel electrophoresis of *in vitro* translation products following transcription of RNA from genomic-length MBV cDNA clone pMBVFLC (C1), pMBV-2 (the second AUG codon in ORF1 altered to GCG, C2), pMBV-3 (the first AUG codon in ORF1 altered to UAG, C3), or pMBV-4 (both AUGs altered, C4). The sizes of ^{14}C-labeled molecular weight markers are indicated (Mw). (B) Analysis by gel electrophoresis and RIP of *in vitro* translation products precipitated by antiserum directed against the polypeptide encoded by ORF1 [lanes 1–4, presented in the same order as (A) above], or ORF2 (lane 5, C1; lane 6, C4). The sizes of the polypeptides (kDa) are indicated.

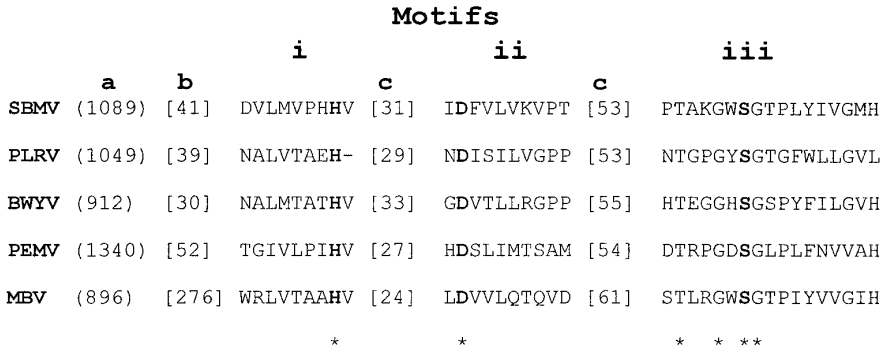

FIG. 4. Alignment of the chymotrypsin related serine protease motifs encoded by MBV ORF2 with those of a number of plant viruses, as defined by Koonin and Dolja (1993). The amino acids of the putative catalytic triad identified by Gorbalenya *et al.* (1989) are highlighted in bold type. The nucleotide number in the genomic sequence of the first base encoding the start of motif (i) is shown (a). The number of amino-acid residues from the start of the ORF is shown (b), as is the number of residues between motifs (c). The identical amino acids are indicated by an asterisk. SBMV, southern bean mosaic virus; PLRV, potato leafroll virus; BWYV, beet western yellows virus; PEMV, pea enation mosaic virus; MBV, mushroom bacilliform virus.

ORF1 prevented synthesis of the M_r 21,000 polypeptide (lane 3); however, removal of the second AUG in ORF1 (lane 2) had no detectable effect on translation. No polypeptide of M_r 21,000 was detected when both AUGs were removed (lane 4).

To identify the translation products encoded either by ORF1 or ORF2, radioimmunoprecipitations (RIPs) were performed using specific antibodies raised against bacterial fusion proteins containing either ORF1 or ORF2.

Polypeptide encoded by ORF1

Three lines of evidence indicated that the M_r 21,000 polypeptide produced following *in vitro* translation of genomic-length RNA transcripts corresponded to the polypeptide encoded by ORF1. First, antiserum raised against the ORF1-encoded fusion protein immunoprecipitated the M_r 21,000 polypeptide (Fig. 3B, lanes 1 and 2). Second, the size of the ORF1 product (starting at nt 61) calculated from the deduced amino acid sequence is M_r 20,000, close to the size determined by gel migration. Third, removal of the first AUG at nt 61 in ORF1 prevented the synthesis of the M_r 21,000 polypeptide (Figs. 3A, lanes 3 and 4, and 3B, lanes 3 and 4).

A second polypeptide of M_r 19,000 was also immunoprecipitated by the anti-ORF1 antiserum (Fig. 3B, lanes 1–4). Initiation at the second AUG in ORF1 at nt 100 would produce a protein of M_r 18,500. However, removal of this AUG, either alone (Fig. 3B, lane 2), or in combination with the first ORF1 AUG (Fig. 3B, lane 4), did not prevent production of the M_r 19,000 polypeptide. This suggested that the M_r 19,000 polypeptide was not produced by translation from the second AUG but was an unrelated polypeptide that was also immunoprecipitated by the ORF1 antiserum. Thus the origin of the M_r 19,000 polypeptide remained unexplained.

Polypeptide encoded by ORF2

The size of the M_r 72,000 translation product (Fig. 3A, lanes 1–4) estimated by gel migration was consistent with that predicted for the polypeptide encoded by ORF2 (M_r 73,000). The product was also immunoprecipitated by antiserum directed against the amino terminal portion of the ORF2 protein, as were two additional polypeptides of M_r 48,000 and M_r 50,000 (Fig. 3B, lanes 5 and 6). These polypeptides may be either ORF2-related proteins or nonspecific precipitates, since faint bands corresponding to polypeptides of these sizes were also seen after RIP with ORF1 antisera (Fig. 3B, lanes 1–4). Removal of both flanking ORF1 AUGs had no apparent effect on translation of the ORF2 product (Figs. 3A, lane 4, and 3B, lane 6).

The detection of the lower-molecular-weight proteins and the location of VPg within the ORF2 protein suggested that the ORF2 protein is cleaved. A candidate for the active protease is the ORF2 protein itself, since it contains chymotrypsin-like serine protease motifs identified by Koonin and Dolja (1993) and shows some similarity to the putative proteases of southern bean mosaic virus (SBMV), pea enation mosaic virus (PEMV), and the subgroup II luteoviruses PLRV and beet western yellows virus (BWYV) (Fig. 4). However, replacement of the putative catalytic serine residue (and an adjacent conserved glycine residue) with alanine using site directed mutagenesis had no detectable effect on the products of *in vitro* translation immunoprecipitated by the ORF2 antiserum (data not shown).

DISCUSSION

It has previously been shown that the 5′ genome arrangement of MBV is similar to some plant viruses, particularly subgroup II luteoviruses, PEMV, and the cowpea strain of SBMV (Revill *et al.,* 1994; Mäkinen *et al.,*

1995; Revill, 1995). In addition, the amino-acid sequences for the putative serine protease and RNA-dependent RNA polymerase (RdRp) genes for these viruses are similar to MBV (Revill *et al.,* 1994; Mäkinen *et al.,* 1995; Revill, 1995). Our results confirm that the similarity to plant viruses extends to the initiation of protein synthesis *in vitro* at the 5′ end of the MBV genome where translation of a second ORF is initiated downstream of the start point for the first ORF and to the location of the coding sequence for the VPg, which is the same as for the subgroup II luteovirus PLRV.

Translation of MBV ORF1 and ORF2 commences at the first AUG in each reading frame, even though the preferred Kozak sequence for initiation of translation (xA/Gxx*AUG*G; Lütcke *et al.,* 1987; Kozak, 1989; Matthews, 1991) is not present for ORF1 (xUxx*AUG*A) and is only partially satisfied for ORF2 (xAxx*AUG*U). This arrangement may facilitate initiation of translation at the second AUG (leaky scanning). Other examples exist of plant viruses with overlapping genes at the 5′ end of genomic RNA, in which the fidelity of the Kozak sequence is stronger for the second AUG than the first. These include subgroup II luteoviruses (Mayo *et al.,* 1989; Veidt *et al.,* 1992), PEMV (Demler and de Zoeten, 1991), and tymoviruses (Weiland and Dreher, 1989). In the subgroup II luteoviruses PLRV and BWYV, products of the expected size from both ORFs were also observed after *in vitro* translation of genomic RNA (Mayo *et al.,* 1989; Veidt *et al.,* 1992).

Removing the initiating codon in ORF1 had no apparent effect on the amount of ORF2 polypeptide produced (Fig. 3A, lanes 3 and 4), consistent with the findings of Weiland and Dreher (1989), who showed that mutating the ORF1 AUG of turnip yellow mosaic tymovirus had no effect on cell-free translation of ORF2. Thus for both viruses, initiation of translation at the first AUG *in vitro* is not a significant impediment to initiation at the second.

The observed MBV ORF1 product of M_r 21,000 and the ORF2 product of M_r 72,000 corresponded closely to the predicted M_r of polypeptides encoded by ORF1 and ORF2 (M_r 20,000 and M_r 73,000; Revill *et al.,* 1994). The numerous minor polypeptides ranging in size from M_r 18,000 to 60,000 (Fig. 3A) were possibly due to either premature termination of translation, or the use of truncated RNA molecules as template, or posttranslational proteolysis. We were unable to demonstrate any *in vitro* protease activity that could be ascribed to the motifs for the putative serine protease in the ORF2 protein. Mutagenesis of the predicted catalytic serine residue did not change the polypeptide pattern observed. It is unlikely that the mutated residue was incorrectly chosen, since the primary sequence G-X-S-G for MBV is common in other proteases where serine is the active residue (Dougherty and Semler, 1993); however, serine protease activity has not been reported for subgroup II luteoviruses and sobemoviruses—the viruses most similar to MBV based on amino-acid comparisons. If the putative virus-encoded protease was not active *in vitro*, then the polypeptides of M_r 50,000 and 48,000 may have arisen from one of the processes mentioned above, with any proteolysis due to a host enzyme. However, during virus replication in infected cells, excision of VPg from the ORF2 protein apparently occurs, and the best candidate for this is the putative ORF2 serine protease.

The MBV ORF2-encoded putative serine protease, and those of sobemoviruses, subgroup II luteoviruses, and PEMV, are similar to the $3C^{Pro}$ cysteine proteases of picornaviruses but are unusual for plant viral proteases in having a nucleophilic serine residue instead of cysteine in the catalytic triad, similar to cellular serine proteases (Gorbalenya *et al.,* 1988, 1989; Dougherty and Semler, 1993; Koonin and Dolja, 1993). An analysis of the known cleavage sites used by these enzymes enables predictions to be made about the residues at the termini of other polypeptides they cleave, such as the VPg of MBV.

Picornaviral $3C^{Pro}$ proteases cleave primarily at E(Q)/S(G) sites and less frequently at E/T sites (Gorbalenya *et al.,* 1988, 1989; Wellink and van Kammen, 1988). In MBV, an E/S dipeptide is present at the VPg N terminus (nt 1334, Fig. 1). Cleavage at a downstream E/T dipeptide (nt 1757, Fig. 1) would result in an M_r 16,300 polypeptide, which is similar in size to the two VPg-related species detected in these experiments (M_r 15,000 and 16,000). Alternatively, cleavage at one of two Q/S sites downstream of the E/T site (nts 1780 and 1792, Fig. 1) would produce proteins of M_r 17,000 or M_r 17,500, respectively, again similar in size to the observed polypeptides.

The location of the MBV VPg downstream of the putative protease motifs encoded by ORF2 is the same as for the VPg of the subgroup II luteovirus PLRV (van der Wilk *et al.,* 1997). However, the VPgs share no sequence similarity and are markedly different in size, with an M_r of 7,000 for the PLRV VPg (Mayo *et al.,* 1982), compared to M_r 15,000 to 16,000 for MBV. However, the MBV VPg is similar in size to that reported for another subgroup II luteovirus, barley yellow dwarf virus (BYDV, approximate M_r 17,000) (Murphy *et al.,* 1989), although the location of the coding sequence for the BYDV VPg is yet to be determined. The shared genome arrangement (protease-VPg-polymerase) of MBV and PLRV is unusual, as all single-stranded RNA viruses for which a VPg has been identified have the arrangement VPg-protease-polymerase (Koonin and Dolja, 1993; van der Wilk *et al.,* 1997).

The similarity of the MBV and PLRV genome arrangements suggests related expression strategies for the two viruses. Luteoviruses have small genomes (approximately 6 kb) and use several means of gene expression that have been demonstrated experimentally. These include internal initiation/leaky scanning, posttranslational proteolysis, subgenomic RNA, ribosomal frameshifting, and stop codon readthrough (reviewed by Matthews, 1991; Miller *et al.,* 1995). The results presented in this paper show that MBV (i) expresses both ORF1 and ORF2, at

least in a cell-free translation system, most likely by leaky scanning, and (ii) codes for a VPg which is probably released by internal proteolysis of the ORF2 polyprotein. Other mechanisms of gene expression have not been demonstrated experimentally, although the similarity of the MBV genome arrangement to that of subgroup II luteoviruses suggests it is likely that the coat protein (ORF4) is translated from a subgenomic RNA, and the putative viral polymerase (ORF3) is synthesised as a fusion protein with the ORF2 protein by a −1 translational frameshift (Revill *et al.,* 1994). Further studies are now underway using fungal protoplasts to determine the MBV translation strategy *in vivo* and to elucidate whether MBV can replicate in the absence of other viruses linked with La France disease.

MATERIALS AND METHODS

DNA manipulations were performed essentially as described by Sambrook *et al*. (1989), except where otherwise indicated. All plasmids were cloned into *Escherichia coli* strain DH5α. PCR was performed using a Corbett Research FTS-320 Thermal Sequencer, with the following conditions: (i) 2 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min; (ii) 28 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min; and (iii) 1 cycle of 94°C for 30 s, 50°C for 30 s, and 72°C for 5 min. DNA was sequenced on both strands of all constructs using a PRISM™ Dyedeoxy™ Terminator Cycle Sequencing kit and an automated sequencer (Applied Biosystems). Nucleotide numbering refers to the MBV genome (Accession No. U07551, Revill *et al.,* 1994).

Analysis of VPg

Commercially grown *A. bisporus* sporophores displaying symptoms typical of LFd (obtained from the same farm as that used for earlier experiments; Revill *et al.,* 1994) were used as the source of virus. MBV was purified from sporophores using the method of Tavantzis *et al*. (1980), and stored at −70°C. RNA was extracted from frozen MBV particles with a Qiagen RNeasy kit according to the manufacturer's instructions. Protein attached to 5 μg RNA was iodinated using chloramine T and 100 μCi [^{125}I] (2000 Ci/mmol) (Greenwood *et al.,* 1963). The ^{125}I-labeled protein and RNA were treated with either 200 μg/ml RNase A or 1 mg/ml proteinase K (in 10 mM Tris-Cl, 500 μM EDTA, pH 7.5) at 37°C for 30 min. The products of digestion were electrophoresed through a preformed 16.5% Tris-Tricine polyacrylamide gel (Bio-Rad), and VPg was detected by autoradiography.

The N-terminal amino acid sequence of unlabeled VPg was obtained using Edman degradation; the starting material was VPg linked to 200 μg MBV RNA obtained from purified virions as described above, and samples were prepared for analysis using the method of van der Wilk *et al*. (1997).

Genomic-length cDNA

To construct genomic length MBV cDNA, the 5′ and 3′ termini of MBV were amplified from appropriate cDNA clones by PCR and ligated to a 3.8-kb MBV cDNA fragment (nts 9–3809) contained in plasmid pMBV19. The steps were as follows.

The 5′ end (114 nt) was amplified from a 5′ cDNA clone (Revill *et al.,* 1994), using an upstream primer (5′GAGGATATC*TAATACGACTCACTATA*GACAAAATAATTGAAG3′) containing an *Eco*RV site, a T7 RNA polymerase promoter sequence (italics), an additional G nucleotide for efficient initiation of transcription, and the first 15 nucleotides of MBV sequence (bold type). The downstream primer was complementary to the MBV sequence (nts 114–84, 5′CAGACCATGAGACATAGCCTTACACTCGTCG3′). The PCR product was blunt ended and phosphorylated using the procedure of Starr and Quaranta (1991) and cloned into *Sma*I-digested pUC18 (Pharmacia). The insert was removed by digestion with *Eco*RV and *Nru*I (nt 82) and ligated into pMBV19 to produce pMBV195′.

The 3′ end of the MBV genome was amplified from cloned cDNA (Revill *et al.,* 1994) using an upstream primer (nts 3378–3402, 5′GAGTTGAGTTCACTGATGTCCAGGC3′) and a 3′ terminal primer incorporating sequential *Sac*II and *Hin*dIII restriction sites, and the last 49 nucleotides of MBV sequence (bold type) (5′CCGCGGAAGCTTACCCAGCCACTCCAAAAGAAATTTCTTTCTTTATGGAGCGACCTTCGAC). The PCR product was cloned into pUC18 as described above, sequenced, and the region containing the MBV 3′ terminus excised by *Mun*I (nt 3457) and *Sac*II digestion. The fragment was cloned into pMBV195′, and the complete genomic-length fragment incorporating the T7 promoter sequence was excised by *Hin*dIII digestion and cloned into pUC18 to generate plasmid pMBVFLC.

Site-specific mutagenesis

ORF1. Three constructs containing mutations in one or both of the first two AUGs in MBV ORF1 were produced (Fig. 1, constructs 2–4). DNA (nts 36–422) was amplified by PCR using gene-specific oligonucleotides incorporating the desired nucleotide change(s) and cloned into *Sma*I-digested pUC18. The DNA was then digested with *Bgl*II (nt 40) and *Xho*I (nt 287), and the fragment was cloned into plasmid pMBVFLC. Construct 1 refers to the parental and unmutated genomic-length cDNA (pMBVFLC). In construct 2, the second AUG in ORF1 (nt 100) was altered to GCG, leaving the first AUG (nt 61) intact (pMBV-2). For construct 3 (pMBV-3), the first AUG in ORF1 (nt 61) was altered to UAG. For construct 4, pMBV-2 served as template DNA, enabling both AUGs to be altered to UAG and GCG, respectively (pMBV-4).

ORF2 encoded protease

To determine whether the putative serine protease encoded by MBV ORF2 was active *in vitro*, the putative catalytic serine residue (Gorbalenya *et al.,* 1988, 1989; Dougherty and Semler, 1993; Revill, 1995) and adjacent glycine (conserved in subgroup II luteoviruses and MBV, Fig. 2.) were both altered to alanine using the overlap PCR method (Ho *et al.,* 1989). The DNA incorporating the desired nucleotide changes was cloned into *Sma*I-digested pUC18, then excised by digestion with *Stu*I and *Xho*I and ligated into plasmid pMBVFLC. Following cloning into *E. coli*, the mutated plasmid was known as pMBVPro.

Production of fusion proteins and antisera

DNA corresponding to nucleotides 60–600 of MBV was amplified by PCR using cDNA as template, and flanking primers designed to introduce *Bam*HI and *Eco*RI sites at the 5′ and 3′ ends, respectively. The PCR product was cloned into the *Bam*HI/*Eco*RI sites of pGEX3X (producing pORF1–3X) and pGEX2T (producing pORF2–2T) (Pharmacia). These plasmids have restriction sites in different reading frames, such that expression of pORF1–3X in *E. coli* produced a polypeptide comprised of glutathione-S-transferase (GST) fused to the complete ORF1 gene product, and expression of pORF2–2T produced a polypeptide corresponding to GST fused to the 145 amino terminal residues of ORF2. Following sonication and pelleting of the cell debris, the soluble fraction was analysed by polyacryalmide gel electrophoresis (PAGE), and polypeptides of the predicted size for MBV ORF1 and ORF2 products fused to GST were excised from the gel. Antisera to these polypeptides were raised by injecting rabbits with macerated gel extracts and bleeding at regular intervals, using standard protocols (Harlow and Lane, 1988).

In vitro transcription and translation of genomic-length constructs

Plasmid pMBVFLC (5 μg DNA) and mutant constructs were linearised with *Sph*I, leaving 8 nt of non-MBV sequence and a 3′ overhang at the 3′ end of the MBV genomic-length cDNA. The DNA was blunt-ended with the Klenow fragment of DNA polymerase, and RNA was transcribed using T7 RNA polymerase (Ribomax™ kit, Promega) in the presence of 1 mM of the cap analog $m^7G^{(5')}ppp^{(5)}G$ according to the manufacturer's instructions. The concentration and integrity of the RNA was determined by electrophoresis through an agarose gel containing formaldehyde and ethidium bromide staining. Following phenol/chloroform extraction and ethanol precipitation, 1 μg of RNA was translated for 90 min in the presence of 30 μCi of translation grade [^{35}S]methionine (1175 Ci/mmol, ICN Pharmaceuticals), using rabbit reticulocyte lysates or wheat germ extracts (Promega). Products were analysed by electrophoresis through 14% polyacryalamide gels and autoradiography.

Radioimmunoprecipitation analysis

The translation mix (10 μl) was added to 100 μl lysis buffer (Smith and Wright, 1985), containing 1 mg/ml Pefabloc (Boehringer Mannheim). The lysate was pre-absorbed with 50 μl protein A Sepharose (Pharmacia) for 1 h at 4°C, cleared by microcentrifugation, and 4 μl of antisera was added and mixed for 12–16 h at 4°C. The immunoprecipitate was bound to 50 μl protein A Sepharose for 1 h at 4°C, collected by microcentrifugation, washed (Smith and Wright, 1985), and analysed by electrophoresis through 14% polyacryalamide gels and subsequent autoradiography.

ACKNOWLEDGMENTS

We acknowledge the support of the Australian Research Council in providing the funds for this research. Our thanks also go to Dr. Frank van der Wilk for helpful suggestions regarding the isolation of the VPg.

REFERENCES

Demler, S. A., and de Zoeten, G. A. (1991). The nucleotide sequence and luteovirus-like nature of RNA 1 of an aphid non-transmissible strain of pea enation mosaic virus. *J. Gen Virol.* 72, 1819–1834.

Dougherty, W. G., and Semler, B. A. (1993). Expression of virus-encoded proteinases: Functional and structural similarities with cellular enzymes. *Microbiol. Rev.* 57(4), 781–822.

Goodin, M. M., Schlagnhaufer, B., and Romaine, C. P. (1992). Encapsidation of the La France disease-specific double-stranded RNAs in 36nm isometric virus-like particles. *Phytopathology* 82, 285–290.

Gorbalenya, A. E., Donchenko, A. P., Blinov, V. M., and Koonin, E. V. (1989). Cysteine protease of positive-strand RNA viruses and chymotrypsin-like serine proteases. *FEBS Lett.* 243, 103–114.

Gorbalenya, A. E., Koonin, E. V., Blinov, V. M., and Donchenko, A. P. (1988). Sobemovirus genome appears to encode a serine protease related to cysteine proteases of picornaviruses. *FEBS Lett.* 236, 287–290.

Greenwood, F. C., Hunter, W. M., and Glover, J. S. (1963). The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. *Biochemistry J.* 89, 114–123.

Harlow, E., and Lane, D. (1988). "Antibodies. A laboratory Manual." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Ho, S. N., Hunt, H. H., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989). Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 77, 51–59.

Hollings, M. (1962). Viruses associated with dieback disease of cultivated mushrooms. *Nature* 196, 962–965.

Hollings, M., Stone, O. M., and Atkey, P. T. (1968). Mushroom viruses. *Rep. Glass. Crop. Res. Ins.* 1967, 101–102.

Koonin, E. V., and Dolja, V. V. (1993). Evolution and taxonomy of positive-strand RNA viruses: Implications of comparative analysis of amino acid sequences. *Crit. Rev. Biochem. Mol. Biol.* 28, 375–430.

Kozak, M. (1989). The scanning model for translation: An update. *J. Cell Biol.* 108, 229–241

Lütcke, H. A., Chow, K. C., Mickel, F. S., Moss, K. A., Kern, H. F., and Scheele, G. A. (1987). Selection of AUG codons differs in plants and animals. *EMBO J.* 6, 43–48.

Mäkinen, K., Tamm, T., Naess, V., Truve, E., Puurand, U., Munthe, T., and Saarma, M. (1995). Characterisation of cocksfoot mottle sobemovirus

genomic RNA and sequence comparison with related viruses. *J. Gen. Virol.* 76, 2817–2825.

Matthews, R. E. F. (1991). "Plant Virology," 3rd ed. Academic Press, California.

Mayo, M. A., Barker, H., Robinson, D. J., Tamada, T., and Harrison B. D. (1982). Evidence that potato leafroll virus RNA is positive-stranded, is linked to a small protein and does not contain polyadenylate. *J. Gen. Virol.* 59, 163–167.

Mayo, M. A., Robinson, D. J., Jolly, C. A., and Hyman, L. (1989). Nucleotide sequence of potato leafroll luteovirus RNA. *J. Gen. Virol.* 70, 1037–1051.

Miller, W. A., Dinesh-Kumar, S. P., and Paul, C. P. (1995). Luteovirus gene expression. *Crit. Rev. Plant. Sci.* 14, 179–211.

Murphy, J. F., D'Arcy, C. J., and Clark, J. M. J. (1989). Barley yellow dwarf virus RNA has a 5′-terminal genome-linked protein. *J. Gen. Virol.* 70, 2253–2256.

Revill, P. A. (1995). La France disease of Agaricus bisporus: The characterisation of mushroom bacilliform virus and the detection of double-stranded RNA. PhD. dissertation. Monash University, Clayton, Victoria, Australia.

Revill, P. A., Davidson, A. D., and Wright, P. W. (1994). The nucleotide sequence and genome organisation of mushroom bacilliform virus: A single stranded RNA virus of *Agaricus bisporus* (Lange) Imbach. *Virology* 202, 904–911.

Romaine, C. P. (1995). *Barnaviridae.* Virus Taxonomy. Sixth report of the International Committee on taxonomy of viruses. *Arch. Virol. Suppl*. 10, 483–484.

Romaine, C. P., and Schlagnhaufer, B. (1995). PCR analysis of the viral complex associated with La France disease of *Agaricus bisporus. Appl. Environ. Microbiol.* 61, 2322–2325.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Smith, G. W., and Wright, P. J. (1985). Synthesis of proteins and glycoproteins in dengue type 2 virus-infected Vero and *Aedes albopictus* cells. *J. Gen. Virol.* 66, 559–571.

Starr, L., and Quaranta, V. (1991). An efficient and reliable method for cloning PCR-amplification products: A survey of point mutation in integrin cDNA. *BioTechniques* 13, 612–618.

Tavantzis, S. M., Romaine, C. P., and Smith, S. H. (1980). Purification and partial characterisation of a bacilliform virus from *Agaricus bisporus*: A single stranded RNA mycovirus. *Virology* 105, 94–102.

Tavantzis, S. M., Romaine, C. P., and Smith, S. H. (1983). Mechanism of genome expression in a single-stranded RNA virus from the cultivated mushroom, *Agaricus bisporus. Phytopathol. Zeit.* 106, 45–50.

Van der Lende, T. R., Harmsen, M. C., and Wessels, J. G. H. (1994). Double-stranded RNAs and proteins associated with the 34 nm virus particles of the cultivated mushroom *Agaricus bisporus. J. Gen. Virol.* 75, 2533–2536.

Van der Wilk, F., Verbeek, M., Dullemans, A. M., and Van den Heuvel, J. F. J. M. (1997). The genome-linked protein of potato leafroll virus is located downstream of the putative protease domain of the ORF1 product. *Virology* 234, 300–303.

Van Zaayen, A. (1979). Mushroom viruses. *In* "Viruses and Plasmids in Fungi," (P. A. Lemke, Ed.), pp. 239–324. Marcel Dekker, New York.

Veidt, I., Bouzonbaa, S. E., Leiser, R., Ziegler-Graff, V., Guilley, H., Richards, K., and Jonard, G. (1992). Synthesis of full-length transcripts of beet western yellows virus RNA:messenger properties and biological activity in protoplasts. *Virology* 186, 192–200.

Weiland, J. J., and Dreher, T. W. (1989). Infectious TYMV RNA from cloned cDNA: Effects *in vitro* and *in vivo* of point substitutions in the initiation codons of two extensively overlapping ORFs. *Nucleic Acids Res.* 17, 4675–4687.

Wellink, J., and Van Kammen, A. (1988). Proteases involved in the processing of viral polypeptides. *Arch. Virol.* 98, 1–26.